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## Single-Cell Mass Spectrometry of Metabolites and Proteins for Systems and Functional Biology

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### Abstract

Molecular composition is intricately intertwined with cellular function, and elucidation of this relationship is essential for understanding life processes and developing next-generational therapeutics. Technological innovations in capillary electrophoresis (CE) and liquid chromatography (LC) mass spectrometry (MS) provide previously unavailable insights into cellular biochemistry by allowing for the unbiased detection and quantification of molecules with high specificity. This chapter presents our validated protocols integrating ultrasensitive MS with classical tools of cell, developmental, and neurobiology to assess the biological function of important biomolecules. We use CE and LC MS to measure hundreds of metabolites and thousands of proteins in single cells or limited populations of tissues in chordate embryos and mammalian neurons, revealing molecular heterogeneity between identified cells. By pairing microinjection and optical microscopy, we demonstrate cell lineage tracing and testing the roles the dysregulated molecules play in the formation and maintenance of cell heterogeneity and tissue specification in frog embryos (*Xenopus laevis*). Electrophysiology extends our workflows to characterizing neuronal activity in sections of mammalian brain tissues. The information obtained from these studies mutually strengthen chemistry and biology and highlight the importance of interdisciplinary research to advance basic knowledge and translational applications forward.

### Keywords

Single cell; mass spectrometry; functional biology; proteomics; metabolomics; cell and developmental biology; neurobiology; *Xenopus laevis*; zebrafish; mouse

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## 1. INTRODUCTION

Modern ‘omics provide an unprecedented set of technologies to study connections between molecular composition and biological function. By enabling the detection and quantification of genes, transcripts, proteins, peptides, and metabolites, valuable information is gained on the molecular underpinnings of biological processes. High-throughput sequencing routine profiling of gene expression to large populations of single cells [1–3]. Identification and quantification of molecular markers from discovery ‘omics help develop hypotheses and design experiments to evaluate their biological function.

Functional biological experiments leverage diverse types of approaches and technologies. Functional tests borrow tools of molecular biology to knock down or out genes, transcripts, or proteins, e.g., by using transcription or translation blocking morpholinos and CRISPR-Cas9. Electrophysiology allows for eavesdropping on neurons. Different types of microscopies, typically optical to electron, enable characterization of cell morphology, phenotype, and anatomy (Fig. 1). Single-cell RNA-sequencing with single-molecule fluorescence *in situ* hybridization (sm-FISH) recently captured molecular cell heterogeneity in the brain in high spatial and molecular resolution [4], supplementing classical knowledge of brain anatomy with molecular information [5]. By comparing single thalamic neurons projecting to motor, somatosensory, and visual cortices in the mouse brain, this approach uncovered several cell types within each projection. Technologies from modern ‘omics empower classical biology and neuroscience with new investigative capabilities.

Single-cell mass spectrometry (MS) supports systems biological studies with information on the molecular state of cells. It complements single-cell transcriptomics by directly measuring proteomic, peptidomic, and metabolomic composition (Fig. 1). Detection with excellent molecular specificity facilitates identification without the requirement for functional probes. For example, MS does not necessitate antibodies for detection. High sensitivity and a broad linear dynamic range permit quantification of molecules at endogenous concentrations. Stringent reporting guidelines [6,7] and public repositories hosting data for reuse, reanalysis, and exchange (e.g., PRIDE [8] and MetabolomicsWorkbench [9]) promote scientific rigor and accountability in MS-based research. The current state of the field of single-cell MS was the focus of several reviews covering technology and application [10–19]. Single-cell MS is adaptable to broad types of molecules, can be made sufficiently sensitive to quantify physiological concentrations, and is compatible with cells of broad dimensions and types, as well as different model systems used in biology and health studies.

Optical microscopy with single-cell MS integrates morphological and molecular information. Recent reviews provide a comprehensive discussion of single-cell MS [12,18–20]. Figure 1 illustrates our protocol for optically guided single-cell MS in embryos of the South African clawed frog (*X. laevis*) and zebrafish and sections of the mouse brain. Cells were identified, dissected [21,22] or their contents directly microaspirated [23–25] for metabolomic and proteomic analysis using capillary electrophoresis (CE) or liquid chromatography (LC) electrospray ionization (ESI) high-resolution MS (HRMS) (reviewed in [26–29]). Microscopy with single-cell MS enabled detection of ~1,500 proteins in 2- to

50-cell *X. laevis* embryos and orthogonal validation using immunohistochemistry [30,31]. Single-cell proteomics by MS (SCoPE MS) quantified 3,000+ proteins from 1,490 cells [32,33], and a single-cell printer with liquid vortex capture enabled rapid metabolomics (~25 cells/min) [34]. With low attomole sensitivity, HPLC columns of narrow bore dimension or with a stationary phase supported on a monolith or a porous layer open tubular (PLOT) format allowed for the identification of ~1,300–4,000 proteins from 50–200 cells via magnetophoretic isolation from whole blood [35–37]. Nanodroplet processing in one pot for trace samples (NanoPOTS) identified over ~1,500 proteins from 10 HeLa cells and ~2,400 proteins from 100 pancreatic islet cells, supporting profiling across clinical samples [38]. These and other leaps in technology expanded the classical toolbox of cell biology, as was discussed in our recent review of the fields [26–29].

We and others built CE-MS platforms to study biomolecules and their role in cell and neurobiological processes. CE combines advantages for single-cell analyses. The physical dimensions of fused silica capillaries are amenable to the limited amounts of sample that are contained in single cells. CE provides several methods for concentration enrichment in the capillary to boost sensitivity to low-abundance molecules (e.g., reviewed in [39]). An exquisite separation power and various data alignment strategies permit reproducible identifications [40,41]. CE-ESI interfaces offer various designs to help hyphenate CE with HRMS for sensitivity, robustness, and reproducibility [42,43].

These custom-built CE-ESI-HRMS platforms revealed previously unknown details on cellular biochemistry. Proteins or metabolites were measured in single cells of *X. laevis* and zebrafish embryos (Fig. 1 and 2) [21–23,25], single neurons dissected from *Aplysia californica* [44,45] and mouse [46,40], and single HeLa [47] cells. Our CE-ESI interface enabled the identification of hundreds of cationic and anionic metabolites (Fig. 3) [48] and ~700 proteins from ~5 ng protein digest from single *X. laevis* cells [25]. Targeted neuropeptides were detected in record sensitivity in the subfornical organ (SFO) and the paraventricular nucleus (PVN) of the mouse hypothalamus (Fig. 3D) [49]. With a 200-zmol lower limit of detection, this technology also identified ~500 proteins from ~1 ng and ~225 proteins from ~500 pg protein digest, which estimates to a single neuron [50,46,51]. Our second-generation CE-ESI HRMS design employing a microprobe capillary enabled the *in situ* and *in vivo* analysis of single identified cells in live embryos [25,23,52] and the mouse brain [53,54,50] (Fig. 2). As shown in Figure 4, its integration with cell labeling and stereomicroscopy permitted the tracing of tissue developmental trajectory. These instrumental capabilities revealed differences in the proteomic [22,25,55] and metabolic [21,23] state of cells in *X. laevis* and zebrafish embryos, including those occupying the dorsal-ventral [21,22,56], animal-vegetal [25,57], and left-right [58,59] developmental axes in the frog. They also led to discovering metabolite-induced cell fate changes [21,60] and metabolic communication between neighboring cells in *X. laevis* [61]. Further, the approach can be extended to patch-clamp electrophysiology, permitting the metabolomic [62] and proteomic [63,53,54] characterization of identified neurons in the mouse brain. Most recently, we also integrated single-cell CE MS with assays measuring background color preference and swim pattern to assess the impact of metabolic perturbation on organismal behavior [61,52].

This chapter presents our protocol enabling functional biological studies with insights to the proteomic and metabolomic state of cells in chordate embryos and mammalian neurons (Fig. 1). We overview required consumables and instruments (see Materials and Methods) and discuss the experimental workflow (Fig. 2). After identifying cells based on optical microscopy or electrophysiology and cell sampling by dissection or microprobe aspiration, the collected material is processed, and the resulting metabolites, peptides, or proteins are measured using LC and CE. Statistical analysis of signal abundances detected by HRMS–MS/MS allowed us to identify compounds for biological investigations. As an example, we present our approaches to test the effect of select compounds on tissue development (Fig. 4). Representative examples are discussed with references to data showing the integration of single-cell ‘omics (Fig. 3) with functional biology (Fig. 4). The *Note* section advises on troubleshooting from the vantage point of an experimentalist, thus hoping to promote the combined use of single-cell HRMS-based proteomics/metabolomics with functional biology in other cell types and biological models.

## 2. MATERIALS

### 2.1. Culturing Embryos and Neurons

1. Animals: Adult male and female *Xenopus laevis* frogs (e.g., from Nasco, Fort Atkinson, WI); adult male and female zebrafish (e.g., from Zebrafish International Resource Center, Eugene, OR); Adult male mouse (e.g., from Charles River Laboratories, Wilmington, MA) (see Note 1).
2. Equipment: Incubators set to 14 °C and 18 °C; Stereomicroscope.
3. Solutions:
  - a. Dejelling solution (2% cysteine): Dissolve 4 g of cysteine in 200 mL deionized (DI) water. Add 10 M sodium hydroxide dropwise to adjust pH to 8.
  - b. 100% Steinberg’s solution (SS): Mix 3.4 g sodium chloride (NaCl), 0.05 g potassium chloride (KCl), 0.08 g calcium nitrate ( $\text{Ca}(\text{NO}_3)_2 \times 4 \text{H}_2\text{O}$ ), 0.205 g magnesium sulfate ( $\text{MgSO}_4 \times 7 \text{H}_2\text{O}$ ), 0.66 g Trizma hydrochloride, 0.075 g Trizma base in 1 L of DI water. Adjust the pH to 7.4 by adding Trizma base and autoclave the solution. Store at 4–14 °C.
  - c. Anesthetic solution (ketamine, 20 mg/mL and dexmedetomidine, 0.1 mg/mL): Prepare by mixing 200  $\mu\text{L}$  of ketamine stock solution (100 mg/mL) and 200  $\mu\text{L}$  of dexmedetomidine stock solution (0.5 mg/mL) with 800  $\mu\text{L}$  of 0.9% saline solution for injection (e.g., part no. NDC0409-4888-06, Hopira, Inc., Lake Forest, IL).

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Note 1. *X. laevis*, mouse, and zebrafish are sentient and vertebrate animals; therefore, protocols pertaining to the care and handling of the animals must be approved by institutional and federal agencies. The work presented in this chapter was approved by the Institutional Animal Care and Use Committee of the University of Maryland (approval numbers R-DEC-17-57, R-FEB-21-07, and R-JUN-20-31) and/or The George Washington University (approval numbers #A311 and #A283).

- d. HEPES ringer solution: Prepare by mixing the following reagents to the following final concentrations: 86 mM NaCl, 2.5 mM KCl, 1.2 mM sodium phosphate ( $\text{NaH}_2\text{PO}_4$ ), 35 mM sodium bicarbonate ( $\text{NaHCO}_3$ ), 20 mM HEPES, 25 mM glucose, 5 mM sodium ascorbate, 2 mM thiourea, 3 mM sodium pyruvate, 1 mM  $\text{MgSO}_4$ , 2 mM calcium chloride ( $\text{CaCl}_2$ ).
- e. Perfusion solution for mouse brain slices: Prepare by mixing the following reagents to final concentration: 126 mM NaCl, 21.4 mM  $\text{NaHCO}_3$ , 2.5 mM KCl, 1.2 mM  $\text{NaH}_2\text{PO}_4$ , 2.4 mM  $\text{CaCl}_2$ , 1.0 mM  $\text{MgSO}_4$ , and 11.1 mM glucose.

## 2.2. Embryology

1. Equipment:
  - a. Stereomicroscope for embryology to identify, inject, and microsample single cells (e.g., SMZ1270, Nikon, Melville, NY) and phenotype embryos, larvae, and tadpoles (e.g., SMZ18, Nikon).
  - b. Microinjector to inject into or aspirate contents from identified cells (e.g., PLI-100A, Warner Instruments, Hamden, CT).
  - c. Micromanipulator (e.g., MM-33, Warner Instruments)
  - d. Capillary pullers (e.g., P-1000 for fused silica capillaries and P-2000 for borosilicate capillaries, Sutter Instruments, Novato, CA)
  - e. Ancillary equipment: Heat block; Centrifuge (e.g., Refrigerated 5430R, Eppendorf)
2. Materials and Solutions:
  - a. Fine sharp forceps (e.g., Dumont #5, Fine Science Tools)
  - b. Centrifuge vials (e.g., 0.2–0.5 mL LoBind tubes, Eppendorf)
  - c. Borosilicate glass capillary (e.g., 0.5/1.0 mm inner/outer diameter, Sutter Instruments)
  - d. Hair loop: Place both ends of a fine hair (~10 cm length) into a 6-inch Pasteur pipet to form a 2 mm loop. Secure it in place with melted paraffin. Sterilize before use by dipping in 70% ethanol and air-drying.
  - e. Dissection dish: Cover the bottom of a Petri dish (35- or 60-mm) with non-toxic modeling clay (e.g., Claytoon was tested in *Xenopus* labs [64]). Make 3–5 wells of ~1.5 mm diameter using a cool glass bead to hold the embryos in place.
  - f. Injection dish: Cover the bottom of a Petri dish (35- or 60-mm) with non-toxic modeling clay. Make several ~1.5 mm wells using a cool glass bead across the dish to hold ~20–30 embryos.

- g. 50% Steinberg's solution: Dilute 500 mL of 100% SS with 500 mL of DI water.
- h. 20% Steinberg's solution: Dilute 200 mL of 100% SS with 800 mL of DI water.

### 2.3. Sample Processing

1. Equipment: Vacuum concentrator (e.g., CentriVap, LabConco, Kansas City, MO)
2. Reagents: All reagents are LC-MS grade to reduce chemical interference during MS detection. Methanol, anhydrous acetonitrile (ACN), water, acetic acid, 100 mM TEAB, 5% hydroxylamine, isobaric labeling kit (e.g., TMTsixplex, ThermoFisher Scientific or iTRAQ, AB Sciex)
3. Solutions:
  - a. Metabolite extraction solvent: 50% (v/v) methanol in water containing 0.5% acetic acid.
  - b. Proteomic digestion buffer: 50 mM ammonium bicarbonate containing protease inhibitor (1 protease inhibitor cocktail tablet per 10 mL)
  - c. Trypsin (0.5 µg/µL) in 50 mM acetic acid
  - d. Patch-clamp solution: We use 50 mM ammonium bicarbonate in water as a compromise between sensitivity and function.

### 2.4. Mass Spectrometry

1. Instrument and Materials:
  - a. CE system (e.g., laboratory-built following [21,40] or CESI, AB Sciex, Toronto, Canada)
  - b. High-resolution tandem mass spectrometer (e.g., quadrupole time-of-flight, Impact HD or TimsTOF, Bruker Scientific, Billerica, MA or quadrupole orbitrap Q-Exactive Plus or Orbitrap Fusion Lumos, Thermo Scientific, Fig. 1)
  - c. HPLC (e.g., Acquity I-class UPLC, Waters, Milford, MA and Dionex Ultimate 3000, ThermoScientific)
  - d. Separation CE capillary (e.g., 40/100 µm inner/outer diameter fused silica, Polymicro Technologies, Phoenix, AZ)
  - e. LC column (e.g., Acquity UPLC BEH Amide Column, 1.7 µm, 1 mm × 100 mm, and Acclaim PepMap C18 column, 3 µm, 0.075mm × 250 mm, Waters)
2. Solutions: All solvents and reagents are LC-MS grade.
  - a. CE background electrolyte solution (BGE): 1% (v/v) formic acid in water for metabolomics; 25% (v/v) acetonitrile in water with 1 M formic acid for proteomics.

- b. CE-ESI sheath solution: 0.1% (v/v) formic acid and 50% methanol for metabolomics; 10% (v/v) acetonitrile in water with 0.05% acetic acid for proteomics.
- c. LC mobile phase for metabolomics using hydrophilic interaction LC (HILIC): For cationic separation, mobile phase A is aqueous 0.1% formic acid and B is acetonitrile containing 0.1% formic acid; for anionic separation, mobile phase A is aqueous 5% acetonitrile with 10 mM ammonium bicarbonate (pH 9) and B is aqueous 95% acetonitrile with 10 mM ammonium bicarbonate (pH 9).
- d. LC mobile phase for proteomics using reversed-phase LC (RPLC): Mobile phase A is aqueous 0.1% formic acid and B is acetonitrile containing 0.1% formic acid.

## 2.5. Functional Studies

1. Instrument and Equipment:
  - a. Epifluorescence stereomicroscope (e.g., SMZ18, Nikon, Fig. 1)
  - b. Inverted microscope (e.g., Eclipse Ti-U, Nikon)
  - c. Microinjector (e.g., PLI-100A, Warner Instruments)
  - d. Micromanipulator (e.g., MM-33, Warner Instruments)
  - e. Camera with tripod (e.g., ESO70D, Canon, Fig. 1)
  - f. Software for processing movies (e.g., Windows media player software)
  - g. Ancillary: Incubator set to 14 °C; Nutator rotator
  - h. Patch amplifier for electrophysiology (e.g., Sutter Instruments)
2. Materials:
  - a. Tadpole food (e.g., part no. SA05964, Nasco)
  - b. Transfer pipets
  - c. Black electrical tape
  - d. 26 G needle (e.g., part no. BD305115, Fisher Scientific)
  - e. 1/2-gallon tank (e.g., part no. SB19271M, Nasco)
  - f. Fine sharp forceps (e.g., Dumont #5)
  - g. Inoculating turntable (e.g., part no. 50809-022, VWR)
3. Reagents:
  - a. 200 proof ethanol
  - b. Gentamicin antibiotic (e.g., part no. 17-528Z, Fisher Scientific)

- c. Sylgard 184 silicone elastomer (e.g., part no. NC9285739, Fisher Scientific)
  - d. Ficoll 400 (Sigma-Aldrich, St. Louis, MO)
  - e. Benzocaine (part no. E1501-500G, Sigma-Aldrich)
  - f. 1X phosphate-buffered saline (PBS) (Fisher Scientific)
  - g. Fluorescent lineage tracer (e.g., fluorescent dextran 10,000 MW lysine fixable or mRNA lineage tracer, Invitrogen, Carlsbad, CA)
4. Solutions:
- a. 3% Ficoll in 100 % SS: Prepare by mixing 3 g of Ficoll in 100 mL of 100% SS.
  - b. 4% paraformaldehyde: Prepare by mixing 4 g of paraformaldehyde in 40 mL DI water at 60 °C. Add a few drops of 1 N NaOH to adjust pH to 7.4. Add DI water to a total volume of 100 mL.
  - c. DEPC water: Add 1 mL diethyl pyrocarbonate to 1 L DI water. Autoclave the solution, seal, and store at room temperature.
  - d. 10% benzocaine: Weight out 2 g of benzocaine and place in a glass beaker. Add 20 mL 200 proof ethanol and stir using a magnetic bar in a stirrer.
  - e. 2% benzocaine in 20% SS: Add 2 mL of 10% benzocaine to 8 mL of 20% SS dropwise, ensuring complete dissolution.

### 3. METHODS

#### 3.1. Culturing

This step lays out our methodology to culture *X. laevis* embryos to larvae or tadpoles and primary neurons from the mouse on the basis of established protocols [65,66]. *X. laevis* embryos require additional dejelling step for manipulation and functional experiments, as described below.

##### 1. Frog Embryos

1. Obtain fertilized eggs by gonadotropin-induced natural mating of adult *X. laevis* frogs or *in vitro* fertilization as detailed elsewhere [65]. See Note 1 on working with live vertebrate animals.
2. Remove the jelly coat from fertilized eggs as follows:
  - a. Remove excess media from dishes containing fertilized eggs.
  - b. Add dejelling solution and keep embryos unperturbed for 2 min.
  - c. Gently swirl dishes over a 2 min period and immediately decant excess dejelling solution.



- d. Transfer the embryos to a 250 mL beaker and add 10% SS. Gently swirl for ~30 s and decant excess liquid.
  3. Rinse embryos four times with 10% SS to remove remaining dejellying solution.
    - a. Transfer ~300–500 embryos into individual 100 mm Petri dishes containing 100% SS. Place dishes in the 14 °C incubator (see Note 2).
  4. Collect two-cell embryos:
    - a. Under a stereomicroscope, identify 2-cell embryos that display stereotypical pigmentation to accurately mark the dorsal-ventral axis, in reference to established cell fate maps [67].
    - b. Place the selected 2-cell embryos into a 100 mm Petri dish containing 100% SS and incubate at 14 °C until the desired developmental stage.

## 2. Brain Section

1. Brain sections were collected following established protocols [68]:
  - a. Anesthetize male mice aged postnatal day (PND) 21–35 with an intraperitoneal injection of anesthetic solution and perfuse with ice-cold HEPES ringer solution.
  - b. After perfusion, dissect the brain rapidly in horizontal slices (220  $\mu\text{m}$ ) prepared in HEPES ringer solution using a vibratome.
  - c. Recover slices for 1 h at 34°C in oxygenated HEPES holding solution. Then, place slide in the same solution at room temperature until use.

### 3.2. Sample Collection from Single Cells

The goal of this step is to collect material from targeted single cells. In what follows, we give an example for *Xenopus*. The workflow starts with the identification of single cells in the *X. laevis* embryo in reference to established cell fate maps [69,70]. As shown in Figure 2, our laboratory established orthogonal strategies to collect single-cell samples. We dissected identified whole cells from *X. laevis* embryos [21,26] or used fabricated microcapillaries as microprobes to aspirate portions of single identified cells from the embryo [23,28,24] or electrophysiologically characterized mouse neurons [63,53].

#### Whole-Cell Dissection from *X. laevis* Embryos

1. Under a stereomicroscope, identify the cell of interest based on stereotypical cleavage and pigmentation, then dissect it manually as follows:
  - a. Transfer the embryo into a dissection dish containing 50% SS.
  - b. Using a hair loop, place the embryo of interest in a groove.

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Note 2. Development is temperature-dependent in *Xenopus* [77,84], thus providing a helpful tool to time biological and chemical experiments. Low temperatures slow down the speed of cell-cleavage, extending the time to select embryos for experiments.

- c. Gently remove the vitelline membrane surrounding the embryo using sharp forceps.
  - d. Use forceps to hold the embryo, preferably at the opposite side of the cell of interest. Lightly pull away the selected cell from the rest of the embryo.
2. Transfer the isolated cell using a pipet into a LoBind Eppendorf vial containing chilled 10  $\mu$ L methanol ( $\sim$ 4  $^{\circ}$ C) or digestion buffer (see Note 3).
  3. Cool the Eppendorf vial (on ice) to preserve sample at low temperature and store samples at  $-80$   $^{\circ}$ C until analysis and up to 3 months without detectable degradation.

### 1. *In Situ/Vivo* Microsampling

1. Fabricate the microprobe by pulling a borosilicate glass capillary to create a fine tip. We use a capillary puller (P-1000 Sutter Instrument) with custom settings: heat = 355, pull = 65, velocity = 80, time = 150.
2. Using sharp forceps, cut the needle tip to an aperture of  $\sim$ 10–20  $\mu$ m (see Note 4).
3. Mount the microprobe into a capillary holder on a three-axis micromanipulator and connect its distal end to a microinjector.
4. Transfer *X. laevis* embryos into an injection dish containing 50% SS. This protocol does not require removal of vitelline membrane.
5. Use a stereomicroscope to aid viewing and manipulation of the embryo. Use a hair loop to immobilize the embryo of interest into a well in preparation for microsampling. Identify the cell of interest following protocols established elsewhere [69,70].
6. Using a micromanipulator, guide the tip of the microprobe into the targeted cell to pierce through the membrane of the cell. Withdraw  $\sim$ 10–15 nL (or as needed) volume from the targeted cell by applying negative pressure to the microprobe using the connected microinjector.
7. To end the microsampling, reduce pressure and retract the microprobe from the cell.
8. Transfer the collected content into a LoBind Eppendorf vial containing 4  $\mu$ L of metabolite extraction solvent or digestion buffer (see Note 5). We usually inject

Note 3. For metabolomics, place cells in ice-cold 100% methanol immediately upon collection. Methanol denatures enzymes and low temperatures slow down chemical reactions, thus minimizing metabolic changes.

Note 4. Capillaries with too large or too small diameters challenge microsampling or microinjection. In *Xenopus*, we find apertures larger than  $\sim$ 20  $\mu$ m tend to cause substantial damage to the cell membrane. Without the membrane being able to heal, the cytoplasm may leak into the media and the cell may not be able to continue division for functional experiments. Conversely, apertures below  $\sim$ 10  $\mu$ m may clog with yolk and cytoplasmic content, requiring refabrication of the microprobe.

Note 5. Tailor the composition of the metabolite extraction solvent to the type of metabolites of interest in a particular study. To study polar metabolites, we use aqueous 40% (v/v) acetonitrile with 40% (v/v) methanol as the extraction solution. Theoretical predictions based on partition and distribution coefficients can help experimental design [58].

the collected material from the capillary by inserting the tip into the solvent/buffer and applying a positive pressure pulse.

9. Cool the Eppendorf vial (on ice) to preserve sample at low temperature and immediately process the samples via metabolomics or proteomics workflows to prevent molecular degradation.

### 3.3. Sample Processing for MS-Based 'Omics

This section discusses protocols to process the collected materials for HRMS analysis. We use LC and CE to separate biomolecules in complementary performance prior to ESI-HRMS.

#### 1. Metabolomics Workflow

1. Extract metabolites from dissected single cells [21,23] or aspirates collected by microprobe sampling [23,48] as follows:
  - a. For dissected single cells: Retrieve the single-cell samples stored in 100% methanol from  $-80\text{ }^{\circ}\text{C}$  freezer and vacuum-dry them at  $4\text{ }^{\circ}\text{C}$ . Add  $10\text{ }\mu\text{L}$  metabolite extraction solvent. Vortex-mix the vials for 30 s at room temperature to facilitate cell lysis and extraction of metabolites. Sonicate the sample vials for 3 min in an ice bath, followed by vortex-mixing for 1 min at room temperature.
  - b. For microprobe aspirated samples: Retrieve single-cell samples collected in metabolite extraction solvent preserved on ice. Vortex-mix the vials for 1 min at room temperature to facilitate extraction of metabolites.
  - c. For CE-ESI-HRMS, centrifuge the samples (dissected or microsamples) for 5 min at  $8,000 \times g$  at  $4\text{ }^{\circ}\text{C}$  to pellet cellular debris. Proper pelleting is important to avoid CE capillaries from getting clogged. We usually store the aliquot together with the pelleted debris to avoid sample losses. The samples are kept at  $-80\text{ }^{\circ}\text{C}$  until analysis.
  - d. For HILIC-ESI-HRMS, centrifuge the samples at  $13,000 \times g$  for 10 min at  $4\text{ }^{\circ}\text{C}$  and transfer the supernatant into a microvial and vacuum-dry the samples at  $4\text{ }^{\circ}\text{C}$ . Reconstitute the samples in  $10\text{ }\mu\text{L}$  95% (v/v) acetonitrile in water and centrifuge the samples at  $13,000 \times g$  for 10 min at  $4\text{ }^{\circ}\text{C}$  to pellet potential debris that could clog the column (see Note 6). Transfer the supernatant into an LC vial and store the sample at  $-80\text{ }^{\circ}\text{C}$  until analysis.

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Note 6. Debris may clog CE capillaries or LC columns. We find that centrifugation of samples before analysis prevents clogging. The efficiency of centrifugation depends on the field force and time of centrifugation. We typically use  $13,000 \times g$  for 10 min.

## 2. Proteomics Workflow

1. Lyse the collected cell or aspirate by sonication for 5 min. Heat the sample to 60 °C for ~15 min to denature proteins. We usually skip reduction and alkylation steps for our single cell samples for higher sensitivity [25].
2. For one-step digestion, add ~50 ng of trypsin protease to the protein extract and incubate the mixture at 37 °C for 5–6 h. For neurons yielding less starting protein amounts, add ~2 ng trypsin and digest at 60 °C for 1 h.
3. Vacuum-dry the resulting protein digest and store it at –80 °C until analysis.
4. (Optional) To enable multiplexing relative quantification, barcode the dried protein digests. We use TMT isobaric labeling following the vendor's protocol (see Note 7), downscaled to the total amount of protein/peptide contained in the sample:
  - a. Reconstitute the dried protein digest in 10 µL of 100 mM TEAB and tag it with 1 µL of 85 mM TMT reagent.
  - b. Incubate each sample for 1 h at room temperature.
  - c. Quench the reaction with 2 µL of 5% hydroxylamine and incubate the mixture for 15 min at room temperature.
  - d. Mix the multiple tagged samples, vacuum-dry the mixture, and store it at –80 °C for up to 1 month until analysis.

### 3.4. High-Resolution Mass Spectrometry

In this step, biomolecules in the resulting samples are separated and detected using ESI-HRMS. We present protocols for separation based on partition chromatography (LC) and electrophoresis (CE). These separation techniques provide complementary benefits in sensitivity, throughput, and molecular coverage (Fig. 3). The resulting data are processed using established approaches in bioinformatics, including but not limited to statistics, multivariate data analysis (e.g., principal component analysis and hierarchical cluster analysis), or machine learning (e.g., Trace [71,72]). These technologies and related protocols (Fig. 2) allowed us to document metabolic and proteomic differences between cells in embryos of *X. laevis* and zebrafish [58,21,22,25] and single neurons in the mouse brain [53,54,63].

#### 1. CE-ESI-HRMS

1. Construct the CE-ESI interface following protocols established by us and others [40,50,73,44]. A simplified procedure to build a blunt-tip CE-ESI interface follows:
  - a. Cleave a 1-m long piece of fused-silica CE capillary.

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Note 7. To select the spectral resolution appropriate for the multiplexing relative quantification, refer to instructions from the manufacturer of the reagents.

- b. On the outlet end of the CE capillary, burn off ~1.5 mm of polyimide coating and clean using isopropanol. Before proceeding to the next step, ensure that the capillary end is clean of burned residues to avoid the leaching of interfering ion signals from the burned residue.
  - c. Feed the CE capillary outlet-end into a T-junction connected to a sheath-flow capillary.
  - d. Mount the CE capillary into the T-junction to feed the CE capillary through the blunt tip emitter, allowing the capillary to protrude ~40–50  $\mu\text{m}$  past the emitter.
2. Hydrate the CE capillary by flushing with LC-MS grade water overnight.
3. Position the tip of the CE-ESI interface ~5 mm from the inlet orifice of the mass spectrometer.
4. Fill CE capillary with BGE and flush sheath-flow capillary with sheath solution.
5. Initiate the electrospray as follows:
  - a. Using a translation stage, fine-position the electrospray emitter tip ~2–3 mm from the mass spectrometer orifice to operate the electrospray in the cone-jet regime (ESI voltage 1.8–2.0 kV).
  - b. Monitor the electrospray using a stereomicroscope.
  - c. Observe the stability of total ion current (TIC) for ~10–15 min to ensure stable operation before analyzing a sample (see Note 8).
6. Inject ~10–15 nL from the metabolite or protein extract hydrodynamically into the CE capillary following previously described protocols [24,40].
7. Gradually increase CE separation voltage from ground (0 V) to ~20–22 kV. Sudden application of high voltage may break the capillary.
8. Load the MS method as described in Table 1 (see CE-HRMS) and start data acquisition (see Note 9).

## 2. LC-ESI-HRMS

### For Metabolomics:

1. We use the following LC parameters: column temperature, 35 °C; autosampler temperature, 4 °C; injection volume, 1  $\mu\text{L}$ ; flow rate, 130  $\mu\text{L}/\text{min}$ . Positive ion mode gradient: 0–0.5 min 95% B, 0.5–10 min 95–40% B, 10–13 min 40% B, 13–15 min 40–95% B, 15–22 min 95% B; Negative ion mode gradient: 0–0.5 min 99% B, 0.5–2.5 min 99–82.5% B, 2.5–6.5 min 82.5–68% B, 6.5–10 min 68–30% B, 10–13 min 30% B, 13–15 min 30–99% B, 15–22 min 99% B.

Note 8. We consider the electrospray to be stable when the total ion current exhibits less than ~15% relative standard deviation over ~40 min of separation.

Note 9. Tailor MS–MS/MS experimental settings to the chosen separation technology. For example, we adjust the number of targeted molecular features and the duration of dynamic exclusion depending on typical peak widths and the complexity of the sample in LC and CE experiments (see Table 1).

2. Select MS method parameters described in Table 1 (see LC-HRMS).

#### For Proteomics:

1. LC parameters: 0–5 min 2% B, 5–85 min 2–35% B, 86–90 min 70% B, 91–120 min 2% B; autosampler temperature, 4 °C; injection volume, 1 µL; flow rate, 300 nL/min.
2. Load the MS method parameters and start data acquisition. Our typical parameters are listed in Table 1. Adjust ion source settings to get a stable nanospray. (see Note 8 and 9).

### 3. Data Processing

#### Metabolomics:

1. Survey the MS–MS/MS data for molecular features (signals with unique  $m/z$  and separation time) using available software packages. For example, we employ MetaboScape Version 4.0.4 (Bruker Daltonics) using the following settings: intensity threshold, 1,000 counts; minimum peak length, 5 spectra.
2. Annotate metabolites based on the accurate mass, isotopic distribution pattern, and tandem MS spectra against reference spectra available in MS–MS/MS databases, including but not limited to METLIN [74], EMBL (<http://curatr.mcf.embl.de/>), MzCloud (<https://www.mzcloud.org/>), MassBank of North America (<https://mona.fiehnlab.ucdavis.edu/>), and the Human Metabolome Database [75]. For example, we use METLIN with an annotation tolerance 10 ppm mass accuracy and MS/MS score 700–900.
3. Perform relative/absolute quantification using under-the-peak-areas (label-free quantification) or ion signal abundances (multiplexing quantification) serving as a proxy for metabolite abundance.
4. Perform statistics and multivariate data analysis to select molecules for follow-up functional studies (see Fig. 2).

#### Proteomics:

1. Identify proteins using established bioinformatics software packages broadly available for bottom-up proteomics. For example, we analyze the MS–MS/MS data in ProteomeDiscoverer (Thermo Fisher Scientific) or MaxQuant (Max Planck Institute of Biochemistry) against the mouse or *Xenopus* proteome (e.g., downloaded from UniProt [76] or Xenbase [77]) with the following search parameters: digestion enzyme, trypsin; missed cleavages, up to 2; variable modification, methionine oxidation; precursor mass tolerance, 20 ppm; fragment mass tolerance, 4.5 ppm; minimum peptide length, 5. Peptides are filtered to <1% false discovery rate (FDR), calculated against a reversed-sequence decoy database. The reported proteins are grouped based on the closest parsimony principle. We remove common contaminants from the final list of protein identifications by manually annotating for common contaminant proteins (downloaded from UniProt).

2. Employ label-free or label-based strategies and software packages from the proteomics community to compare protein levels between single cells. For example, we used MaxQuant Version 1.5.5.1 [78] or Proteome Discoverer (Thermo Scientific) to quantify the proteomic state of single embryonic cells and neurons by calculating label-free quantitative indexes (LFQ values) [57,25] or relative reporter ion signal abundances from TMTs [22].

### 3.5. Functional Studies

The goal of this step is to link chemistry with biological function. As an example, we describe a protocol to prepare brain tissues to record neuronal activity followed by *in vitro* single-cell proteomics [63,53]. In the context of cell differentiation, we trace cell fates to understand how cells divide to form specific tissues and organs. Because cell fates are reproducible in *X. laevis* [79,80], it is possible to inject molecules into identified cells to determine their developmental impact on tissue specification and organogenesis [21,60,61]. Figure 4 shows an example, in which the vehicle or select metabolites were injected into specific cells, while fluorescently monitoring their tissue clone via the co-injection of the green fluorescent dextran. Alternatively, fluorescent proteins can be expressed in the cell, for example, by injecting the corresponding mRNA [64,21]. In *X. laevis* tadpoles, it is also possible to perform behavioral assays to assess sensory (e.g., visual), muscular, cognitive, and other functions. As an example, we adopted the background color preference assay [81] to test the behavior of pre-metamorphic *X. laevis* tadpoles after performing *in vivo* single-cell MS on the precursor embryo [52].

#### 1. Single-Neuron Electrophysiology and Capillary Microsampling

1. Perfuse midbrain slices continuously at 1.5–2 mL/min with perfusion solution at 28–32 °C, following established protocols [82].
2. Backfill the patch pipettes for recording (2–4 M $\Omega$ ) with ~20  $\mu$ L of 50 mM ammonium bicarbonate in water (see Note 10).
3. Putatively identify neuron type of interest. For example, dopaminergic neurons can be identified based on their location in the lateral portion of the substantia nigra and their size. Consult with brain anatomy atlases to improve the accuracy of tissue identifications.
4. Neuron identification may be aided by electrophysiology. Detection of a slow pacemaker firing pattern (> 2 ms action potential), indicates dopaminergic neurons [83].
5. Obtain a giga-ohm seal and record action potential for 60 s in a cell-attached configuration. A patch-clamped neuron is demonstrated in Fig. 2. We used the Sutterpatch software to control the devices [53,54,63].

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Note 10. It is imperative to optimize the composition of the intracellular solution used for patch clamp electrophysiology to the osmolarity of the neuron. Although potassium gluconate is commonly used in electrophysiological recordings, we use ammonium bicarbonate to minimize spectral and ionization interferences during MS caused by involatile salts.

6. Following electrophysiological analysis, apply a steady negative pressure at the outlet end of pipette with a syringe to aspirate a portion of neuronal soma (see Note 11).
7. Under an inverted microscope (40× magnification), visually inspect the neuron during microaspiration. A slight reduction in neural soma size is anticipated from successful patching. Figure 2 shows a neuron during sampling after electrophysiological recording.
8. Gently withdraw the pipette from the cell then expel the collected contents into an LoBind microvial containing 5  $\mu$ L protein digestion solution chilled on ice. After a 1 h digestion at 60 °C, store samples at –80 °C for up to 1 month without detectable degradation, until analysis.

## 2. Cell Lineage Tracing

### Cell Labeling by Microinjection

1. Prepare fluorescent tracer solution:
  - a. Prepare a 100  $\mu$ L solution containing 0.5% fluorescent dextran in DEPC water.
  - b. (Optional) Synthesize capped mRNA using an *in vitro* transcription kit. Prepare a working solution containing ~50–100 pg/nL mRNA pellet in DEPC-treated water.
2. Prepare injection needles following steps described earlier. Calibrate the volume of injection by injecting water droplet into mineral oil and measuring the diameter of the water droplet.
3. Fill the injection needle with ~0.5–1  $\mu$ L fluorescent tracer solution using a microinjector in “fill” mode following established protocols [64].
4. Transfer the embryo into an injection dish containing 3% Ficoll in 100% SS.
5. Under a stereomicroscope, use a hair loop to gently angle the embryo to orient the cell of interest for facile access for injection (see Fig. 4).
6. Using a calibrated micromanipulator, guide the tip of the injection capillary into the targeted cell. Inject ~1–5 nL of the sample by applying +40 psi on the capillary for ~300–500 ms using a microinjector (see Note 12).
1. Culture the injected embryos in 3% Ficoll in 100% SS for 3–4 h to allow the cell membrane to heal. Transfer the embryos to 50% SS and culture at 14–22 °C until the larval stages 32–34 (see Note 13).

Note 11. Aid sample collection by applying consistent negative pressure and continuously monitoring the size of the neuron under the inverted microscope. Shrinking neuronal soma is an indication of successful sampling. Be careful not to aspirate the media surrounding the neuron to avoid sample dilution and interferences due to salt during MS analysis.

Note 12. Limit damage to the cell membrane by carefully withdrawing the needle tip from the cell. With negligible damage, the cell membrane heals, and the embryo continues development. Damage to the cell membrane can cause leakage of cytoplasmic content, which can result in low survival rates or lethality. Take extra care to also avoid damaging the neighboring cells to facilitate development.

Note 13. Based on our experience and other protocols [80], culturing at lower temperature (14–16 °C) improves survival rates.



## Fixing and Imaging of the Larvae/Tadpoles

1. Anesthetize the larvae or tadpoles on ice or using 0.5% benzocaine in 100% SS. Ensure the success of anesthesia by gently touching the tadpole with a capillary, anticipating no response if successful. Increase the concentration of benzocaine if necessary. Ensure tadpoles are handled and treated humanely so that the organisms do not suffer or feel pain (see Note 1).
2. Fix the specimens in 4% paraformaldehyde for 1 h on a rotator.
3. Rinse the tadpoles with 1X PBS twice. Store the tadpoles in 1X PBS at 4 °C.
4. For imaging, mount the specimens in a 30 mm dish containing ~1–2 mL of 1X PBS. Image the specimens using epifluorescence microscopy (e.g., SMZ18, Nikon) (see Note 14).
5. Acquire images using a microscope following the manufacturer's instructions.
6. Conduct lineage analysis by determining the relative contribution of fluorescent cells to tissues and organs (see protocols in References [69,70,79]).

## 3. Behavioral Assay

### Tadpole Preparation

1. Obtain and culture embryos as described earlier.
2. Inject identified cells with the test compounds as described earlier. Prepare negative control by injecting identified cells with DEPC-treated water. Use non-injected embryos for later use as the wild-type control group.
3. Place the embryos in the 14 °C incubator until they reach gastrula stage. Transfer the embryos to room temperature in 90 mm Petri dishes containing 20% SS and change media every two days and culture the tadpoles until the feeding stage (Stage 45) (see Note 1).
4. Feed tadpoles every other day as follows:
  - a. Mix food with 20% SS solution to form a paste.
  - b. Place the paste in a corner of the Petri dish containing the tadpoles.
  - c. Provide more food as needed.
5. Maintain the tadpoles under a 12 h light/dark light cycle.

### Background Color Preference Assay

1. The color preference assay is performed in a setup consisting of nested tanks following an established protocol [81]. The inner tank holds the tadpoles. The outer tank provides the background colors with half covered with a black tape

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Note 14. Clearing agents (e.g., benzyl alcohol:benzyl benzoate, BA:BB) may be used in *X. laevis* embryos and tadpoles to improve fluorescent imaging [85]. Tissue clearing is recommended for imaging deep in intact embryos/tadpoles and sections.

and the other half covered with a white paper. Ensure both tanks are water-leveled to aid visual inspection of tadpole behavior.

2. Fill the inner test tank with 20% SS to the 5 cm water mark from the top and insert the inner tank inside the outer tank.
3. Mount the camera on a tripod to record the entire tank from above (see Note 15).
4. Transfer a single tadpole on the white background of the inner tank. Limit this experiment to one tadpole at a time to avoid interactions between tadpoles which may confound behavioral phenotypes.
5. Record the swim pattern of tadpole for 2 min.
6. After 2 min, carefully lift the outer tank, rotate the outer tank, and return the inner tank into the outer tank. This step helps minimize the impact environmental factors may have on behavior. Start recording immediately and set the timer for 2 min.
7. Place the tadpole back to a holding tank containing 20% SS. Record 2 trials for each of ~10–15 tadpoles.
8. Repeat the assay on the next day on the same tadpoles to test for reproducibility and enhance statistical evaluation of the results by obtaining more data.

#### Euthanasia

1. Place tadpoles in a 90 mm Petri dish containing 2% benzocaine in 20% SS for ~15–20 min.
2. Monitor the tadpole's reflex by gently touching with a hair loop. Anesthetized tadpole cannot swim or respond to mechanical stimuli (gentle touching). Only proceed to the next step if the tadpole is anesthetized.
3. Freeze the larvae/tadpoles at  $-20^{\circ}\text{C}$  overnight.
4. Dispose of the tadpoles following protocols approved by the relevant institutional and federal authorities (see Note 1)

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Note 15. The locations of the eyes in tadpoles help accurately determine crossings between the white and black backgrounds (see Fig. 4). Therefore, the camera used to record video trials should have sufficient optical resolution and frame rate to clearly identify the eyes and monitor fast tadpole swimming. We typically use ~30 fps to monitor tadpoles during both the background color preference and swim assays.

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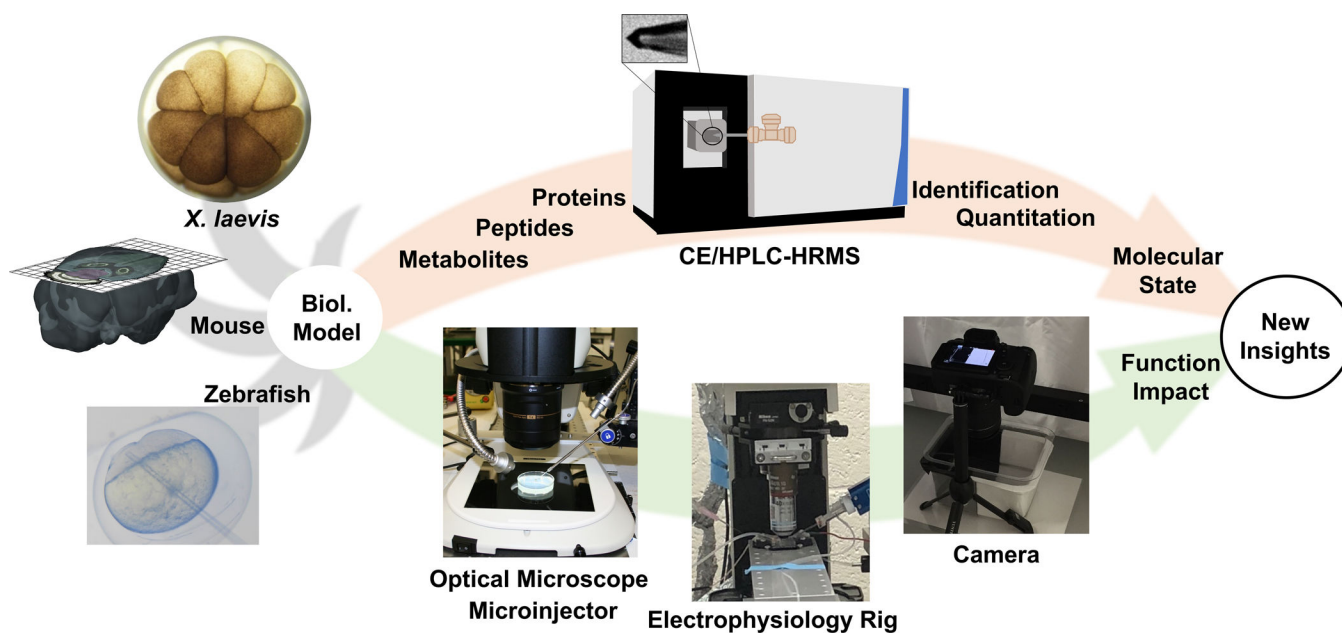
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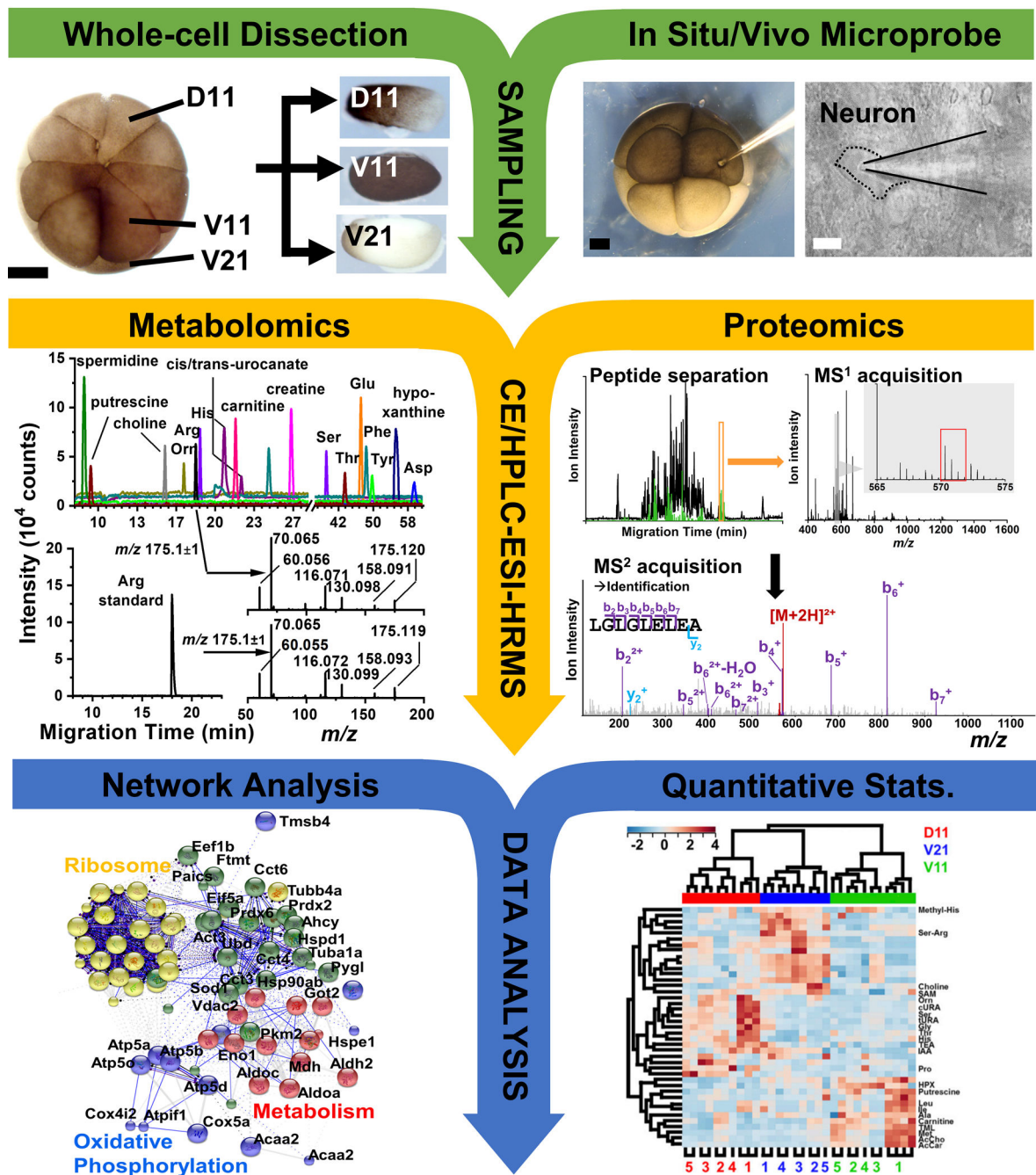
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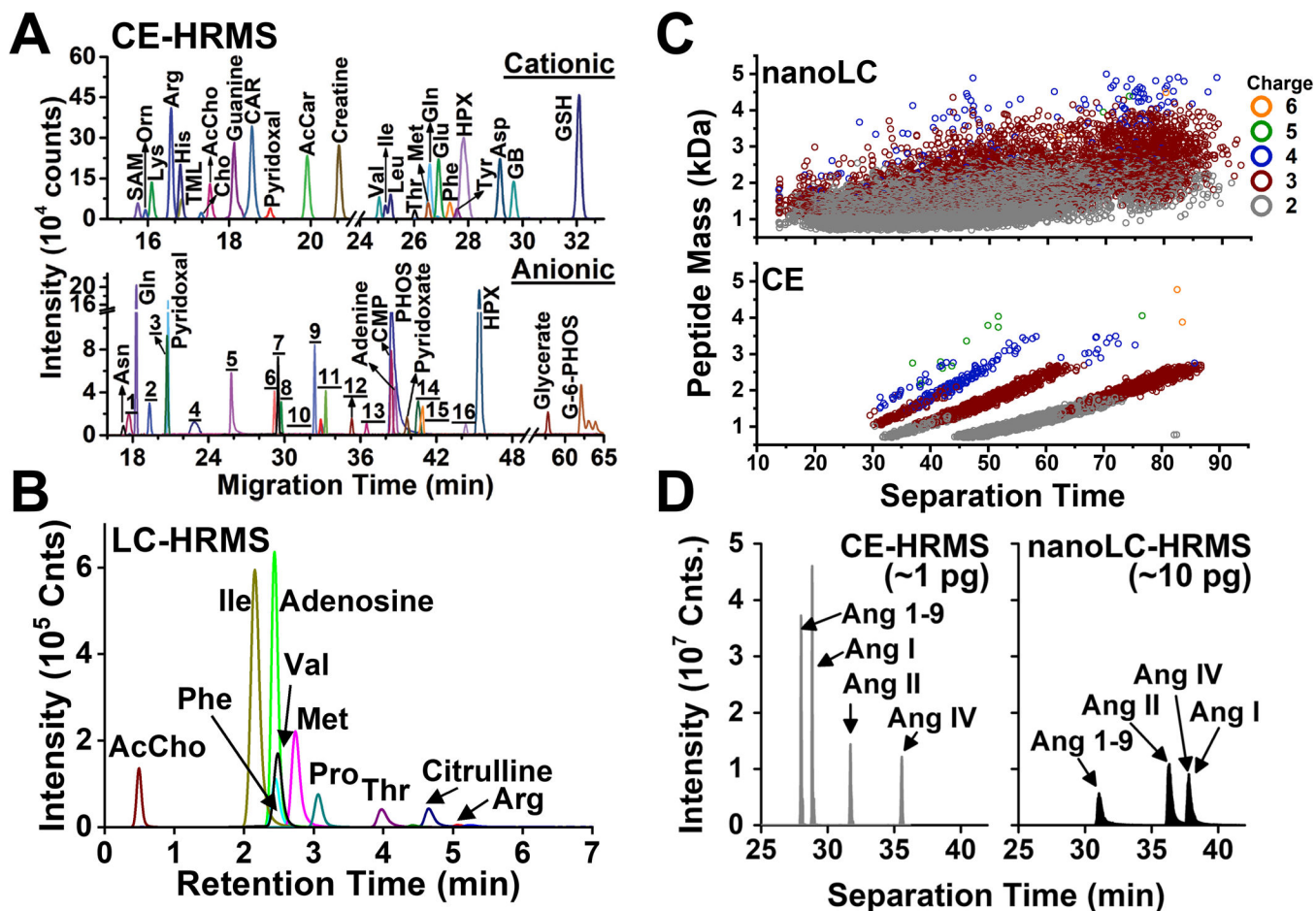


**Figure 1.** Our approach for assessing chemistry and functional in biological models. The protocols discussed in this chapter were developed and validated using embryos from the frog *Xenopus laevis* and zebrafish and sections from the mouse brain. (Adapted with permission from Anal. Chem. 2017, 89, 13, 7069–7076. Copyright 2017 American Chemical Society. Adapted with permission from Anal. Chem. 2019, 91, 7, 4797–4805. Copyright 2019 American Chemical Society.)

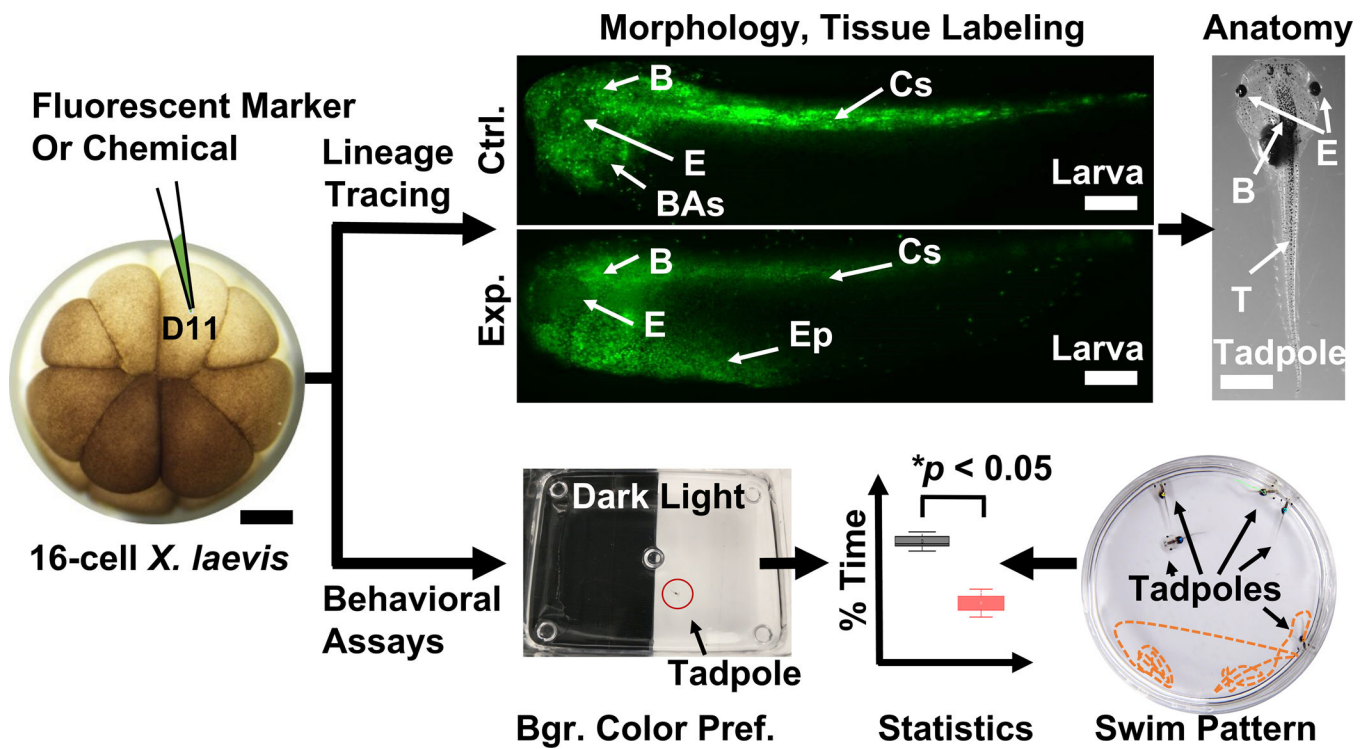


**Figure 2.**

Our general single-cell MS workflow enabling the analysis of metabolites and proteins in single cells of *Xenopus laevis* or zebrafish and single neurons in a section of the mouse brain. Scale bars: 250  $\mu\text{m}$  (black), 20  $\mu\text{m}$  (white). (Adapted with permission from Ref. [57]; Adapted with permission from Ref. [21]. Copyright 2015 National Academy of Sciences; Adapted with permission from Anal. Chem. 2017, 89, 13, 7069–7076. Copyright 2017 American Chemical Society; Adapted with permission from Ref. [26].)



**Figure 3.** Representative detection of metabolites and proteins by CE and HPLC ESI-HRMS. (A) Chemical profiling of anionic and cationic metabolites in a single *X. laevis* cell using CE-ESI-MS. (B) HILIC LC-MS of polar metabolites from limited populations of cells. (C) Comparison of peptide identifications by CE-ESI-HRMS and nanoLC-nanoESI-HRMS. (D) Targeted detection of angiotensin peptides in the PVN and SFO of the mouse hypothalamus using CE and nanoLC. (Reproduced from Ref. [48,49] with permission from The Royal Society of Chemistry and Springer Nature, Copyright 2019.)



**Figure 4.** Techniques to investigate chemistry and function during development. (**Top panel**) Analysis of cell fate, morphology, and anatomy following fluorescence lineage tracing of the left dorsal-animal midline cell (D11) in control (Ctrl.) and experimental (Exp.) *X. laevis* larvae. (**Bottom panel**) Background color preference and swim pattern assays evaluating behavior in *X. laevis* tadpoles. Key: B, brain; BA, branchial arches; Cs, central somites; E, eye; Ep, epidermis. Scale bars = ~250  $\mu\text{m}$  (embryo, larvae), ~1.5 mm (tadpole). (Adapted with permission from Anal. Chem. 2017, 89, 13, 7069–7076. Copyright 2017 American Chemical Society.)

**Table 1.**

Our typical instrumental settings for detecting metabolites and proteins in CE and LC ESI-HRMS using the positive (+) and negative (–) ion mode.

Parameters	CE-ESI-HRMS		LC-ESI-HRMS	
	Q-TOF	Q-OT	Q-TOF	Q-OT
Instrument Configuration	Metabolites	Proteins	Metabolites	Proteins
Compounds	Metabolites	Proteins	Metabolites	Proteins
MS survey scan frequency	MS <sup>1</sup> –MS <sup>2</sup> , 2 Hz	MS <sup>1</sup> , 7; MS <sup>2</sup> , 13 Hz	MS <sup>1</sup> –MS <sup>2</sup> , 2 Hz	MS <sup>1</sup> , 7; MS <sup>2</sup> , 13 Hz
Mass range ( <i>m/z</i> ) and spectral resolution	50–550 at 40,000 FWHM	400–1,700 at 35,000 FWHM for MS <sup>1</sup> and 17,500 FWHM for MS <sup>2</sup>	50–1,300 at 40,000 FWHM	400–1,700 at 35,000 FWHM for MS <sup>1</sup> and 17,500 FWHM for MS <sup>2</sup>
Dry gas	2 L/min (N <sub>2</sub> )	–	4 L/min (N <sub>2</sub> )	–
Nebulizer gas	–	–	0.4 bar (+); 1 bar (–)	–
Dry temperature	100 °C (+); 150 °C (–)	275 °C	220 °C	275 °C
Ion-transfer capillary voltage	–1,700 V (+); +2,100 V (–)	2,300 V	–4,500 V (+); +4,000 V (–)	1,800 V
Collision energy	18 eV (CID)	36% (normalized, HCD)	15–35 eV (CID)	36% (normalized, HCD)
Isolation <i>m/z</i> window	1.5 Da	1.5 Da	1.5 Da	1.5 Da
Dynamic exclusion mass tolerance	5.0 ppm	5.0 ppm	5.0 ppm	5.0 ppm
Dynamic exclusion	9 s	9 s	13 s	13 s
High intensity ion signal threshold (counts)	1.5 × 10 <sup>6</sup>	1.5 × 10 <sup>6</sup>	1.5 × 10 <sup>6</sup>	1.5 × 10 <sup>6</sup>
AGC target (counts)	–	1 × 10 <sup>6</sup>	–	1 × 10 <sup>6</sup>
Minimum AGC target (ion counts)	–	9.2 × 10 <sup>2</sup>	–	9.2 × 10 <sup>2</sup>
Maximum injection time (ms)	–	50	–	50
DDA Top N	5	20	5	20